AMINOPEPTIDASE-P

Arieh Yaron and Daniel Mlynar

Department of Biophysics, The Weizmann Institute of Science,
Rehovot, Israel

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An aminopeptidase of unusual specificity has been found in the bacterial extract of Escherichia coli B. It releases N-terminal amino acids but only if adjacent to proline residues. The enzyme digests high molecular weight substrates as well as low molecular weight peptides. Thus it liberates one mole of isoleucine from one mole of reduced and carboxymethylated papain; degrades high molecular weight poly-L-proline; releases rapidly one mole of arginine and more slowly one mole of proline from bradykinin; it digests peptides such as Gly·Pro·Gly; Pro·Pro·Ala; Pro·Pro·Ala.OMe; Gly·Pro; Ala·Pro; Val·Pro and Pro·Pro; N-terminal proline is not hydrolyzed unless followed by proline. The enzyme requires Mn^{++} (optimum 3.7×10^{-5} M) and has a pH optimum at 8.6. Its molecular weight is about 2×10^5. An isolation procedure, resulting in 860 fold purification has been worked out. The purified enzyme gives one band in acrylamide-gel electrophoresis. The name aminopeptidase-P is suggested for the new enzyme.

Isolation. The enzyme was isolated from Escherichia coli strain B. The cells were ruptured by sonication and the crude extract obtained was subjected to six consecutive purification steps (see Table I, which also gives the assay of enzyme activity). In the first step the crude extract was heated at 50^0 for 15 min causing precipitation of non active proteins. More protein was precipitated by adding $(NH_4)_2SO_4$ to 0.45 saturation and the activity was precipitated from the supernatant by increasing the $(NH_4)_2SO_4$ concentration to 0.64 saturation. The precipitate obtained was dissolved in 0.05 M sodium acetate, pH 5.6 and precipitated with acetone at -3^0. The active protein was then extracted from the precipitate with 0.05 M Na-acetate, pH 5.6. In the following steps Ca-phosphate (Keilin and Hartree, 1938) was used first in portions to effect fractional adsorption. The fraction containing the enzyme was then fractionally eluted with 1.0 M KCl in

Table I. Purification of aminopeptidase-P

Step	Specific Activity* units/mg#	Total Activity* units#	Total Protein** mg	Purification Factor	Recovery of Activity %
Crude enzyme	0.064	6900	107000	1	100
Heat precipitation	0.094	6700	71000	1.5	97
Ammonium sulfate fractionation	0.22	5000	22600	3.4	72
Acetone precipitation	1.2	4520	3780	18.7	56
Ca-phosphate adsorption	8.0	3840	480	125	48
DEAE cellulose	20	2720	134	312	40
Gel filtration	55	2170	39.4	860	31

* The activity was determined as follows: 0.25 ml of poly-L-proline solution (1 mg/ml in water) was mixed with 0.5 ml veronal buffer pH 8.6 and 0.2 ml of a fresh Mn-citrate solution (prepared by mixing one part of each: 0.2 M sodium citrate, 0.1 M manganous chloride, 0.1 M sodium hydroxide and 0.05 M veronal buffer to pH 8.6). The solution was placed into a 40^0 water bath and the enzyme solution (about 0.05 ml containing 10-40 milli-units) was added. After half an hour the reaction was stopped by adding 2.5 ml ninhydrin reagent (prepared by dissolving 3.0 g ninhydride in a mixture of glacial acetic acid (60 ml) and 6 M phosphoric acid (40 ml) at 70^0). For determination of proline formed during hydrolysis, 2.5 ml of glacial acetic acid was added and the solution heated at 100^0 for half an hr. The solution was cooled and the intensity of the red colour formed was measured using a Klett-Summerson photoelectric colorimeter with a filter No. 52.

** Protein concentration was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as standard.

One unit of activity is defined as the amount of enzyme which produces one mg proline per ml of incubation solution under the conditions of the above described assay.

0.05 M Na-acetate pH 5.6 which extracted part of the non active protein, followed by two successive extractions with 0.05 M Na-phosphate, pH 6.0 yielding the 125 fold purified enzyme. In the fifth step the enzyme solution was applied to DEAE-cellulose and the adsorbent washed with 0.05 M Na-acetate, pH 5.6, 0.002 M in sodium citrate and 0.05 M KCl in 0.1 M Na-acetate pH 5.6 containing 0.002 M Na-citrate. The activity was eluted with 0.4 M $(NH_4)_2SO_4$ in 0.1 M sodium acetate, pH 5.6 containing 0.002 M Na-citrate, and emerged in a sufficiently small volume to be applied as such to a 2 m Sephadex

G-200 column. The column was developed with 0.1 M Na-acetate, pH 5.6 containing 0.002 M Na-citrate and the enzyme emerged as an activity peak with constant specific activity over most of its width. The enzyme solution obtained was stable at 4^0 for 9 months, it could not be lyophilized without loss of activity. Concentration of the solution was achieved by applying the sample to a DEAE-cellulose column and eluting it with 0.4 M $(NH_4)_2SO_4$ in 0.1 M Na-acetate containing 0.002 M Na-citrate.

The individual purification steps were followed by acrylamide disc electrophoresis as shown in Fig. 1.

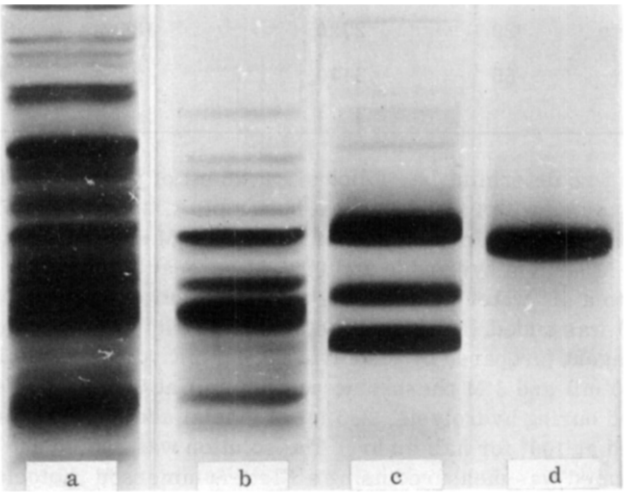

Fig. 1. Polyacrylamide gel electrophoresis (Davis, 1964) of aminopeptidase-P preparations at various stages of purification. a, after acetone precipitation (0.17 activity units); b, after Ca-phosphate adsorption (0.17 activity units); c, after DEAE-cellulose (2.7 activity units); d, after gel filtration (2.7 activity units).

Sedimentation and diffusion coefficients of the pure enzyme were measured in 0.407 M ammonium sulfate, 0.1 M in Na-acetate pH 5.6, containing 0.002 M sodium citrate. The enzyme sedimented as a single symmetric boundary in the ultracentrifuge. From the sedimentation coefficient $s^0_{20,w} = 9.1$ S, obtained after

extrapolation to zero conc., the diffusion coefficient $D^0_{20,w} = 4.4 \times 10^{-7}$ cm^2/sec and an assumed partial specific volume of 0.74, a molecular weight of 205,000 was calculated. A sedimentation equilibrium run with a 0.15 per cent solution of the enzyme in the same solvent evaluated by the Yphantis midpoint method yielded a mol. wt. of 230,000. The enzyme displayed a typical protein absorption peak at 278 mμ and a 1.8 ratio of absorbances at 278 and 260 mμ, respectively. The extinction was $OD_{280} = 1.03$ (1 cm) at a conc. of 1 mg/ml (determined from the Kjeldahl nitrogen using a factor of 6.25).

Mn^{++} requirement. The enzyme requires presence of Mn^{++} ions for its catalytic action. A quite sharp optimum in activity occurs at a Mn^{++} conc. of 3.7×10^{-5} M. This maximum was observed at all pH values at which activity could be tested (7.0 - 9.2). The pH of highest activity was 8.6. The optimal conc. of free Mn^{++} in the assay solution was controlled by using Mn-citrate as a metallo-buffer. Presence of citrate also protected the enzyme against the inhibitory effects of traces of heavy metals. Versene inhibited the enzyme activity completely at a 10^{-3} and 10^{-4} M conc.

Substrate specificity. Aminopeptidase-P acts on the bond between an N-terminal amino acid residue followed by a proline residue: A-Pro-B-C ---.

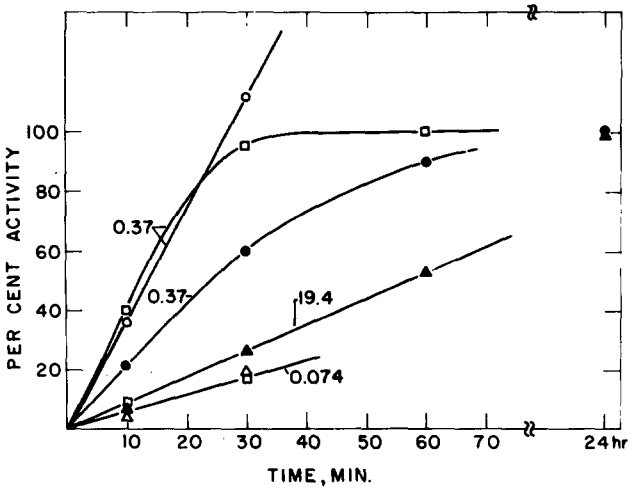

Fig. 2. Substrate specificity. o - o, poly-L-proline (0.33 mM, 30.9 base mM); ◻ - ◻, Pro·Pro·Ala (1.76 mM); ● - ●, Gly·Pro·Gly (1.6 mM); ▲ - ▲, Gly·Pro (2.0 mM). △ - △, Pro·Pro·Ala·OMe (1.70 mM). Incubation conditions were the same as described in Table I for the determination of activity. The enzyme concentrations in μg per ml are given in the Figure.

The terminal amino group is essential, as DNP-polyproline is resistant to hydrolysis. Low mol. wt. peptides hydrolyzed were Pro·Pro·Ala, Pro·Pro·AlaOMe, Gly·Pro·Gly, Gly·Pro (see Fig. 2), Val·Pro, Ala·Pro, and Pro·Pro. It is seen from Fig. 2 that the dipeptide Gly·Pro is digested considerably slower than higher peptides and the same applies to Val·Pro and Ala·Pro. Bradykinin (Arg·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg) was digested rapidly (enzyme conc. 4.4 μg/ml); one arginine being released per one bradykinin molecule in less than five min. The following proline residue was released within one hr (80% in 30 min). No additional splits were observed after 24 hr. Reduced and carboxymethylated papain (which has the N-terminal sequence Ile·Pro·Glu·Tyr·Val - - -) was digested rapidly. At an enzyme conc. of 1.3 μg/ml 1 mole of isoleucine was liberated within 1 hr (substrate: 10^{-4} M). No other amino acid was released. It seems that hydroxyproline cannot substitute for proline in the substrates of aminopeptidase-P since poly-L-hydroxyproline is not digested.

A specific exopeptidase - the proline iminopeptidase - has been isolated and purified from *E. coli* strain K_{12} - W - 1361 which cleaves N-terminal L-proline residues from high mol. wt. substrates such as salmine and poly-L-proline, as well as from low mol. wt. substrates such as L-prolylglycine (Sarid, Berger and Katchalski, 1959, 1962). The absence of proline iminopeptidase in our preparation is shown by the resistance to hydrolysis of salmine and of Pro·Gly.

A substrate specificity similar to that of aminopeptidase-P was repeatedly claimed (Light and Greenberg, 1965; Hill and Schmidt, 1962; Frater, Light and Smith, 1965; Nolan and Smith, 1962) for the dipeptidase prolidase (Davis and Smith, 1957). However, pure prolidase was shown to digest specifically dipeptides with a C-terminal proline or hydroxyproline, whereas the tripeptide Gly·Pro·Gly was not digested (Davis and Smith, 1957). In contrast, aminopeptidase-P digests Gly·Pro·Gly (and bradykinin) much more readily than the dipeptide Gly·Pro.

The observations that prolidase can to some extent cleave glycine from Gly·Pro·Leu (Hill and Schmidt, 1962); isoleucine from denatured papain (Frater, Light and Smith, 1965); glutamine from $GluNH_2$·Pro·Ser·Val·Val·Leu (Light and Greenberg, 1965), and lysine from a peptide Lys·Pro·Arg·Glu - - - (Nolan and Smith, 1962), seem to indicate the presence of aminopeptidase-P in the prolidase preparations, all of which were of low degree of purity.

Aminopeptidase-P extends the specificity of the previously known exopeptidases. For instance, leucine aminopeptidase is capable of removing amino acid residues

until the residue preceding proline. Aminopeptidase-P is capable of cleaving this residue revealing a N-terminal proline, which in turn can be removed by proline iminopeptidase. Thus it is now possible to continue the stepwise hydrolysis with LAP.

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